MARCELA LUMI MIYASAKI

EFICÁCIA DE UM GEL COM REDUZIDA CONCENTRAÇÃO DE FLUORETO SUPLEMENTADO COM HEXAMETAFOSFATO DE SÓDIO SOBRE O PROCESSO DE DESMINERALIZAÇÃO DO ESMALTE DENTÁRIO. ESTUDO *IN VITRO*

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Orientador: Prof. Adj. Alberto Carlos Botazzo Delbem.

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O meu socorro vem do Senhor, que fez o céu e a terra,

Não deixará o teu pé vacilar, aquele que te guarda não dormitará,

Eis que não dormitará, nem dormirá aquele que guarda a Israel.

O Senhor é quem te guarda, o Senhor é a tua sombra á tua mão direita,

De dia o sol não te ferirá, nem a lua de noite,

O Senhor te guardará de todo mal, ele guardará a tua vida,

O Senhor guardará a tua saída e a tua entrada, desde agora e para sempre.

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"Honra teu pai e tua mãe para que prolongue seus dias na terra que o Senhor teu Deus te dá"

Êxôdo 20:12

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Paz e harmonia: Eis a verdadeira riqueza de uma família.

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De tudo ficaram três coisas:
A certeza de que estamos sempre começando...
A certeza de que precisamos continuar...
A certeza de que seremos interrompidos antes de terminar...
Portanto devemos fazer:
Da interrupção um caminho novo...
Da queda, um passo de dança,...
Do medo, uma escada...
Do sonho, uma ponte...
Da procura, um encontro...
E assim terá valido a pena existir!"

(Fernando Sabino)

Miyasaki ML. Eficácia de um gel com reduzida concentração de fluoreto suplementado com hexametafosfato de sódio sobre o processo de desmineralização do esmalte dentário. Estudo *in vitro*. [dissertação]. Araçatuba: Universidade Estadual Paulista; 2014.

Resumo

O objetivo deste trabalho foi avaliar *in vitro* o efeito de gel com baixa concentração de fluoreto suplementado com hexametafosfato na desmineralização do esmalte. Materiais e métodos. Blocos de esmalte bovinos (*n* = 120) foram selecionados baseados na dureza de superfície e divididos entre 6 grupos de tratamento (n=20 por grupo): a) Gel Placebo, sem F ou HMP , b) 9% HMP, c) 4500 µg F/g, d) 4500 µg F/g + 9% HMP, e) 9000 µg F/g e f) 12300 µg F/g (Gel Ácido). Blocos de esmalte bovino foram tratados uma única vez com os respectivos géis. Dez blocos de cada grupo foram analisados quanto à concentração de CaF_2 e F formado . Os outros dez blocos foram submetidos à cinco ciclagens de pH (pH 4,7- 6h e pH 7,0-18h) durante 7 dias. Após as ciclagens de pH, determinou-se a dureza de superfície final (SH1) para o cálculo da porcentagem de perda da dureza de superfície (%SH) e em seguida, a concentração de CaF_2 e F (retido). Subsequentemente, a %SH e perda integrada de subsuperfície (ΔKHN) foram avaliadas. Os resultados foram submetidos à ANOVA seguido do teste Student-Newman-Keuls ($p < 0,05$). Resultados: Os grupos 4500 9%HMP, 9000 e o gel ácido apresentaram menor %SH, os menores valores de perda integrada de subsuperfície foram encontrados nos grupos de 4500 9%HMP e Gel ácido ($p > 0.05$). O grupo de gel de Ácido apresentou maiores concentrações de CaF_2 formado quando comparado aos demais grupos ($p <0,05$). Após ciclagem de pH, o grupo 4500 9% HMP e gel Ácido apresentaram concentrações semelhantes de CaF_2 retidos ($p > 0,05$). A concentração de F formado foi semelhante entre os grupos 4500, 4500 + 9% HMP, 9000 e gel ácido ($p> 0,05$). Não houve alteração na concentração de F retido após a ciclagem de pH nesses grupos. Conclusão: É possível inibir a desmineralização do esmalte com gel de baixa concentração de fluoreto suplementado com 9% HMP. Relevância clínica: O gel com baixa concentração de fluoreto pode ser considerado como uma alternativa mais segura para o uso clínico de um ponto de vista toxicológico, uma vez que contém a metade da quantidade de uma formulação convencional, enquanto promove um efeito anticárie similar.

Palavras-chave: Desmineralização; Fluoretos; Fosfatos.

Miyasaki ML. Efficacy of gels with reduce concentration the fluoride supplemented with hexametaphosphate de sodium in inhibiting demineralization the dental enamel : study *in vitro.* [dissertação]. Araçatuba: Universidade Estadual Paulista; 2014.

Abstract

The objective of this study was to evaluate the in vitro effect of low-fluoride (F) gels supplemented with hexametaphosphate (HMP) on enamel demineralization. Materials and methods: Bovine enamel blocks (*n* = 120) were selected based on surface hardness (SH) and divided into six treatment groups (*n* = 20 per group: a) gel Placebo, b) 9%HMP, c) 4,500 µg F/g d 4,500 µg F/g + 9% HMP, e) 9,000 µg F/g and f) 12,300 µg F/g (Acid gel). Blocks were treated with respective gels only once. Ten blocks of each group were anylized about formed CaF2 concentration. The other ten blocks were subjected to five pH cycling (pH 4,7-6h and pH 7,0-18h) during seven days. After the pH cycling, it was determined the final hardness surface (SH1) to calculate the percentage of loss surface hardness (%SH) and then, the concentration the CaF_2 e F (retained).The results were subjected to ANOVA followed by Student-Newman-Keuls test ($p < 0.05$). Results: The groups 4500 9%HMP, 9000 and Acid showed lower %SH, the lowest values ΔKHN were found for the 4500 9%HMP and Acid gel ($p > 0.05$). The gel acid group showed higher concentrations the CaF_2 formed when compared to the other groups ($p <0,05$). After all cycles of pH, the group 4500 9% HMP and acid gel showed similar concentrations the CaF_2 retained ($p > 0,05$). The concentration the F formed was similar the groups 4,500, 4,500 + 9% HMP, 9,000 and acid gel ($p>0,05$). There was no chance in the concentration the F retained after all cycles of pH these groups. Conclusion: It is possible to inhibit enamel demineralization with low-F gels supplementing these gels with 9% HMP. Clinical relevance: The low-F gel containing HMP can be regarded as a safer alternative for clinical use from a toxicological point of view since it contains half of the amount of a conventional formulation while promoting similar anticaries effect.

Keywords: Demineralization; Fluorides; Phosphates

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Table 1: Mean (SD) values of hardness (n =10) analysis according to the groups

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Figure 1: A - Differential hardness profiles (hardness vs depth) calculated by subtracting no fluoride gels profiles from placebo. B - Differential hardness profiles calculated by subtracting fluoride gels profiles from 4500 group (Table 1).

Lista de abreviatura

LISTA DE ABREVIATURA

Ca^{++}	Íon cálcio
CaF^{+}	Íon Fluoreto de cálcio
CaF_2	Fluoreto de cálcio
$CaHPO_4^0$	Fosfato de cálcio neutro
cm²	Centímetro quadrado
DP	Desvio padrão
F	Fluoreto
g	Grama
h	Hora
HCl	Ácido clorídrico
HF^0	Fluoreto de hidrogênio neutro
HMP	Hexametafosfato de sódio
$H_2PO_4^-$	Íon fosfato di hidrogênio
IH	Dureza interna
K	Potássio
KCl	Cloreto de potássio
KHN	Unidade de dureza Knoop
L	Litro
mg	Miligrama
Min	Minutos
M	Molar
mL	Militro
mm	Milímetro

mm^2	Milímetro quadrado
mol L^{-1}	Mol por litro
mmol/L	Milimol por litro
NaF	Fluoreto de sódio
NaOH	Hidróxido de sódio
P	Fósforo
pH	Potencial de Hidrogênio
s	Segundos
SH	Dureza de superfície inicial
SH1	Dureza de superfície final
TISAB	Tampão ajustador de força iônica total
TMP	Trimetafosfato de sódio
µg	Micrograma
µm	Micrômetro
ΔKHN	Perda integrada de dureza de subsuperfície
%SH	Porcentagem de perda de dureza.

SUMÁRIO

Effect of fluoride gels supplemented with hexametaphosphate in inhibiting demineralization: *in vitro* study

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*** De acordo com as instruções aos autores do periódico Clinical Oral Investigations (Anexo A)**

1.0 Introduction

Professional F application is a well-known method used for dental caries prevention and its efficacy is clearly recognized an evidence-based perspective [Marinho et al., 2002; Villena et al., 2009]. When it is incorporated into the enamel crystal, it forms a fluorapatite like mineral and improves the enamel resistance to the acid challenge [Chow et al., 1990; Moreno et al., 1993]. Products containing high fluoride concentrations are available and have been used in the form of solutions, gels, foams, and varnish [van Rijkom et al., 1998; Marinho et al., 2002]. However caution is needed when using gel in children because of the risk of acute intoxication if the product during application is ingested, and young children do not have fully developed complex sputum [van Rijkom et al., 1998].

One of the possibilities to decrease F ingestion of children is reducing its concentration in gels; however the effectiveness of the gel must be maintained. One method that may be used to increase the effectiveness of low F gels is to supplement with TMP (trimetaphosphate) [Danelon et al., 2013; Danelon et al., 2014]. In studies *in vitro* and *in situ*, Danelon et al. [2013; 2014], associated the TMP and F, resulting in better effect inhibition on demineralization and remineralization of enamel when compared to the gel 12,300 µg F/g, acid gel.

The combination of TMP/F showed great potential as an anticaries treatment [Takeshita et al., 2009, Takeshita et al., 2011] and HMP has the ability to reduce enamel solubility [van Dijk et al., 1980]. Then, it would be interesting to verify whether a similar synergistic anticaries effect exists between fluoride and cyclic HMP [da Camara et al., 2014]. As the complexing ability of a condensed phosphate is proportional to the total number of phosphorus atoms in the

polyphosphate [van Wazer & Campanella, 1950], a minor concentration of fluoride than that utilized by Takeshita et al., 2009 may be added to toothpaste.

A similar inorganic phosphate, hexametaphosphate (HMP), is used extensively in the ceramics industry as deflocculant, because of its strong tendency to form complexes with cations [Andreola et al., 2004; Castellini et al., 2005]. The HMP is used in the food industry as an antimicrobial agent owing to its ability to increase the permeability of the bacterial outer membrane [Vaara & Jaakkola, 1989].

The study of da Camara et al., 2014 concluded that the combination of 0.5% HMP to a toothpaste containing 250 ppm F promotes a similar inhibitory effect on enamel demineralization when compared to a conventional toothpaste with 1,100 ppm F. However, it is observed that no studies evaluating fluoride gels and cyclic HMP on tooth demineralization. More recent studies have shown that a proportion phosphate / fluoride should be objectified suitable for a greater effect caries [Takeshita et al., 2009; Moretto et al., 2010; Amaral et al., 2013; Zaze et al., 2013; Manarelli et al., 2013; Danelon et al., 2013; Favretto et al., 2013; Moretto et al., 2013; Danelon et al., 2014].

Thus far, no studies have evaluated the efficacy of cyclic HMP supplemented low-F gels in combating demineralization. Studies of new gels with these characteristics are important to assess the efficacy of these gels in reducing both enamel demineralization and the risk of intoxication. Therefore, the purpose of the present study was to evaluate the ability of gels with reduced F concentration and HMP supplemented on enamel demineralization, using a pH cycling model. The null hypothesis was that low-F gels associated to HMP would

present the same ability to reduce the enamel demineralization when compared to low-F gel without HMP.

2.0 Material and Methods

2.1. *Experimental Design*

Enamel blocks (4 mm × 4 mm, *n* = 120) of bovine incisors were stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. The enamel surfaces of the blocks were sequentially polished and selected by a surface hardness (SH_1) test and then randomized (320.0 to 380.0 Kgf/mm^2) into 6 groups (*n* = 20 per group): (a) gel without F and HMP (Placebo), (b) gel containing 9% HMP (9%HMP), (c) gel containing 4,500 µg F/g (4500), (d) gel containing 4,500 µg F/g + 9% HMP (4500 9%HMP), (e) gel containing 9,000 µg F/g (9000), and (f) gel containing 12,300 µg F/g (Acid gel). After gel application in 10 blocks from each group, the concentrations of CaF_2 and F formed in the enamel were determined. The other 10 enamel blocks from each group were treated with the respective gels and then subjected to pH cycling for 5 days. After pH cycling, percentage the surface hardness loss (%SH) were calculated. The concentrations of CaF_2 and F retained in the enamel were also determined. And the integrated hardness (IH; KHN × µm) of lesions in sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the IH for sound enamel to obtain the integrated area of the subsurface region in the enamel, which was termed integrated loss of subsurface hardness (ΔKHN; KHN × µm) [Spiguel et al., 2009].

2.2. *Gel formulation and determination of fluoride in products*

Experimental gel of neutral pH was prepared in a laboratory and had the following ingredients: carboxymethylcellulose (Synth, Diadema, São Paulo, Brazil), sodium saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), glycerol (Merck, Darmstadt, Germany), peppermint oil (Synth), and water. F (NaF; Merck) was added to the gel in a concentration of 0, 4,500, or 9,000 μg F/g. Subsequently, HMP (HMP cyclic form, Sigma-Aldrich Co., St. Louis, MO, USA) was added at a concentration of 9% to gels with F concentration of 0 and 4500 μg F/g. A commercial acidic gel was used as a positive control (12,300 μg F/g, acid gel, pH= 4.5, *DFL*; Indústria e Comércio S.A, Rio de Janeiro, RJ, Brazil). The F concentration in the gels was determined using a specific electrode for the F ion (9609 BN; Orion Research Inc., Beverly, MA, USA) attached to an ion analyzer (Orion 720 A^{+}; Orion Research Inc., Beverly, MA, USA) and calibrated with standards containing 0.125 to 2.000 μg F/g. Approximately 100 mg of each product was dissolved in deionized water and transferred to a volumetric flask. The volume was then adjusted to 100 mL using deionized water. For each product, 3 dilutions were made. Subsequently, 2 samples of 1 mL were buffered with total ionic strength adjustment buffer II (TISAB II) [Danelon et al., 2013; Danelon et al., 2014]

2.3. *Gel treatments and demineralization/remineralization cycling*

The treatments were applied for 1 min to each gel block (3 g/block) [Delbem et al., 2002; Delbem et al., 2010; Danelon et al., 2014]. After treatment, the gel was removed, and the blocks were washed with deionized water. Thereafter, 10 blocks

from each group were subjected to five pH cycles at 37°C during a procedure that lasted 7 days [Vieira et al., 2005]. The blocks were then placed in a demineralizing solution (DE: 2.0 mmol/L calcium and phosphate in 75 mmol/L acetate buffer, pH 4.7; 0.04 µg F/mL; 2.2 mL/mm^2). After 6 h, the blocks were transferred to a remineralizing solution (RE: 1.5 mmol/L calcium, 0.9 mmol/L phosphate, and 150 mmol/L KCl in 0.1 mol/L cacodylic buffer, pH 7.0; 0.05 mg F/mL; 1.1 mL/mm^2) for 18 h. The blocks were rinses with deionized water between each step. The blocks were kept in fresh RE solution during the last 2 days of the procedure.

2.4. *Hardness measurements*

The hardness of the enamel surface of each block was determined before and after pH cycling using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25 g load for 10 seconds. Five indentations, spaced 100 µm apart, were created in the center of the enamel block (SH_1). After pH cycling, 5 indentations, spaced 100 µm from the baseline indentations, were created for determination of surface hardness (SH1) to calculated the %SH.

For cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed. They were then gradually polished. One sequence of 14 indentation at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel were created in the central region, using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, IL, USA) with a Knoop diamond indenter under a 5 g load for 10 seconds. The mean values at all three measuring points at each distance from the surface were then averaged. The integrated hardness (IH; KHN × µm) of in sound enamel was

calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the IH for sound enamel to obtain the integrated area of the subsurface region in the enamel, which was termed integrated loss of subsurface hardness (ΔKHN; KHN × μm) [Spiguel et al., 2009]. Hardness measurement can be used to quantify mineral loss since it presents a positive correlation with mineral content [Kielbassa et al., 1999]. To analyze the patterns of demineralization, differential hardness profiles were calculated by subtracting the hardness values of the placebo group from those of the HMP group (i.e., placebo group values minus the 9%HMP group values) and by subtracting the hardness values of the 4,500 group from those of the F and F + HMP groups (i.e., 4,500 group values minus the 4,500 9%HMP, 9,000, and Acid gel group values) at each depth. These differential profiles were then integrated over two depth zones in the lesion (zone A, 5–30 μm; zone B, 40–130 μm) and underlying sound enamel to yield ΔIH values [Danelon et al., 2013; Danelon et al., 2014].

2.5. *Analysis of the CaF_2-like concentration in enamel*

The concentration of CaF_2 in enamel was analyzed to evaluate the amount of CaF_2 and F present after the application of the F gel (CaF_2 and F formed) and after pH cycling (CaF_2 and F retained). A digital caliper (Mitutoyo CD-15B; Mitutoyo Corp., Japan) was used to measure the surface area of the enamel [Caslavska et al., 1975; Delbem et al., 2010; Danelon et al., 2013; Danelon et al., 2014]. The surface of each specimen, except for the enamel, was coated with wax. The specimens were then immersed in 0.5 mL of 1.0 mol/L KOH solution for 24 h under constant agitation. The solution was then neutralized with 0.5 mL of 1.0 mol/L HCl and buffered with 1.0 ml of TISAB II. An ion analyzer (720A; Orion Research, USA)

and a combined ion-selective electrode (9609 BN; Orion Research, USA) previously calibrated with standards of 0.0625, 0.125, 0.250, 0.500, and 1.0 μg F/mL were used. Data were obtained in mV and were converted to microgram F per square centimeter using Microsoft Excel.

2.6. *Analysis of the F concentration in enamel*

After extraction of the CaF_2 and F, an enamel biopsy was performed [Weatherell et al., 1985]. Blocks measuring 2 mm × 2 mm (*n* = 120) were obtained from half of the longitudinally sectioned blocks. To measure depth, the blocks were fixed to a mandrel and attached to a handpiece (N 270; Dabi- Atlante, Ribeirão Preto, SP, Brazil) fixed to the top of a modified microscope with a micrometer (Pantec, Sao Paulo, SP, Brazil). Self-adhesive polishing disks (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50 μm was removed from each enamel block. A total of 0.5 mL of 0.5 mol/L HCl was added to the enamel powder retained on the polishing disk fixed to the polystyrene crystal tube. This mixture was then agitated for 1 h, and then, 0.5 mL of 0.5 mol/L NaOH was added [Alves et al. 2007; Danelon et al., 2013; Danelon et al., 2014]. For the FA-like analysis (FA-like formed and retained), a specific electrode (Orion 9609) was connected to an ion analyzer (Orion 720+) and TISAB II. A 1:1 ratio (TISAB/ sample) was used.

2.7. *Statistical analysis*

For statistical analysis, SigmaPlot software (version 12.0) was used, and the significance limit was set at 5%. Experimental groups were considered as fixed

factors. Data from %SH and ∆KHN were submitted to one-way ANOVA followed by the Student-Newman-Keuls test. The results of CaF_2 and F (log transformation) in enamel were submitted to two-way ANOVA followed by the Student-Newman-Keuls test. The results of ΔIH (considering % of HMP and zone) were submitted to two-way ANOVA followed by the Student–Newman–Keuls test.

3.0 Results

The mean values (SD) of F ion concentration (µg F/g) in the Placebo, 9% HMP, 4500, 4500 9%HMP, 9000, and Acid gel groups were 67.6 (12.4), 88.8 (8.6), 4681.1 (8.6), 4726.4 (145.4), 9067.7 (98.4), and 10900.2 (219.6), respectively. The %SH results (Table 1) showed that the groups 9000, 4500 9%HMP and acid gel had similar hardness values ($p > 0.474$). The ∆KHN results (Table 1) showed that placebo groups and 9% HMP had the highest values when compared to the other groups ($p < 0.05$). The ΔKHN was similar results gels groups 4500 9% HMP and 9000 ($p<0.05$).

Differential hardness profiles (Table 1 and Figure 1) provided evidence of different subsurface lesion patterns among the HMP and F groups. The demineralization process was more pronounced in zone A (5–30 µm, Table 1 and Figure 1) than in zone B among the HMP group ($p < 0.05$). The 4500 9%HMP and Acid gel groups had lower demineralization in zone B (40–130 µm) than did the other groups ($p = 0.001$, Table 1 and Figure 1). Other comparisons are shown in Table 1.

The concentration of CaF_2 formed (Table 2) was higher for all groups 4500 9% HMP, 9000 and acid gel ($p>0.05$). No difference was observed among 4500 9%HMP and acid gel the concentration of CaF_2 retained ($p>0.05$). The group supplemented with fluoride and 9% HMP (Table 2) showed concentrations similar of FA-like formed when compared to fluoride groups ($p>0.05$), and not difference was observed of FA-like retained among groups.

4.0 Discussion

The application of products with high F concentrations is recommended for patients who have a high risk of developing dental caries. The use of these products in young children must be carefully monitored because of the risk of acute intoxication [Fomon et al., 2000; Ripa, 1990]. The purpose of the present study was to evaluate the ability of gels with reduced F concentration and HMP supplemented on enamel demineralization, using a pH cycling model.

Thus, it is important that laboratory studies under controlled and standard conditions be performed to evaluate the anticariogenic potential of new formulations of gels before performing a clinic or *in situ* study. The present study in vitro evaluated the effectiveness of the gel before performing a study *in situ* ou *clinic.* The present study *in vitro* evaluated the efficacy of a gel contained 4500 µg F/g associated to the HMP on concentration of the 9%, showing that this association has a similar efficacy than the gels containing 9000 µg F/g and 12300 µg F/g (acid gel) in reducing the demineralization of enamel, which would allow reduce the concentration of F without changing its effectiveness and therefore, reducing ion intake by children preventing acute intoxication.

The addition of any compound to the fluoride gel should not interfere with the bioavailability of F. The addition of HMP 9% did not alter the concentration of the loss surface hardness (tabela1) in 4500 µg F/g gel (test *t*, p>0,05), what indicates that there was no change in the availability of F.

The choice of the concentration of 9%HMP was based on previous studies of [da Camara et al., 2014], where it was found that for a dentifrice containing 250 µg F/g the best performance in relation to the change of surface hardness of enamel as well as mineral loss was obtained when the toothpaste was supplemented with 0,5% HMP. In the lower or higher concentrations tested the effect was minor. Therefore, for a gel containing 4500 µg F/g to maintaining the same proportion F:HMP, it was supplemented HMP in the concentration of 9%.

The study shows that a gel with 4500 µg F/g is less effective (%SH) than the 9000 µg F/g gel and acid gel (Table1). However, the addition of 9% HMP to the 4500 µg F/g gel promoted an increase of the hardness surface (SH_2) when compared to the not supplemented group, obtaining similar results to the 9000 µg F/g gel and acid gel groups (Table 1).

Therefore, these condensed phosphates on the enamel surface have reduced the mineral loss during the pH cycles, resulting in greater values of final hardness and consequently, lower values of %SH (Table 1). It was observed that in F absent, the group with only 9% HMP showed a lower %SH than the placebo group (Table 1), suggesting that the HMP has an independent action of the F.

By the other hand, the anticariogenic effect of the F high concentration topic products is related to the CaF_2 deposition to the enamel (Hicks et al., 2004; Moreno, 1993), that is concentration and pH dependent (Moreno,1993). The CaF_2

concentration measured soon after the gels application increases with the F concentration present in the gel, being the higher value observed in the acid gel (table 2), followed by the 9000 µg F/g group. The acid pH may also have contributed to the higher formation of CaF_2 in the acid gel (Table 2). The placebo groups and that containing only HMP presented similar values ($p>0.05$) and the lower ones among the groups. Although the group containing only HMP has presented CaF_2 concentration similar to the Placebo, it resulted in a lower %SH than the placebo, showing that this effect does not come from the CaF_2 deposition, possibly by the condensed phosphates protector layer formation on the enamel surface.

However, the supplemented group (4500 9%HMP) %SH was similar than these latest two groups, the 9000 and acid gel showing that other factors are contributing to the inhibition of the demineralization. One of these factors may be related to the incorporated F to the enamel after its application, what must be evaluated in further studies.

In relation to the calcium fluoride that stayed retained after the pH cycles, the results show that possibly the repeated treatments with demineralized solution of pH 4,7 dissolve the calcium fluoride that was formed during the treatment with fluoride gels, so that, in the end of cycles, the final concentration is similar among the 4500 9% HMP and Acid gel groups and the other groups, except the placebo one (table 2). The measured CaF_2 is that is deposited in the enamel surface. The differences in the surfaces hardness among the groups suggest that may be differences in the incorporated F concentration inside the enamel.

The ΔKHN and ΔIH values observed in this study confirm previous findings that HMP reduces mineral loss deep in the enamel (Table 1 and Figure 1) [da Camara et al., 2014].

These complexes provide higher calcium availability during demineralization and remineralization processes, and reduce the precipitation rate of calcium phosphate on the enamel surface. This effect holds the pores on the enamel surface open, facilitating the diffusion of calcium and fluoride ions into deeper regions of enamel. This may explain the effects of 9% HMP added to a 4500 µg F/g gel, but only concentration of 9%HMP, these mechanisms do not seem to apply [Table 1 and Figure 1].

After its adsorption to the enamel surface, the effect of HMP in the presence of fluoride will be retain charged ions of CaF^{+} and Ca^{2+} by replacement of Na^{+} in the cyclic structure. The interaction of Ca^{2+} with one or more HMP molecules leads to the reticular formation [van Wazer & Campanella, 1950] on enamel surfaces also containing CaF^{+}. The hypothesis is that this produces a greater retention of fluoride and calcium with great impact on the ionic activity of charged and neutral species. Under acidic conditions, HMP would release CaF^{+} and Ca^{2+} species to medium, which would subsequently react with $H_2PO^{-}_4$, increasing the ionic activity of neutral ions $CaHPO_4^{0}$ and HF^{0} [Cochrane et al., 2008] mainly during the remineralization phase in the inner part of the lesion. The diffusion coefficient of $CaHPO_4^{0}$ and HF^{0} into enamel lesions is thousand fold higher that of ionic calcium [Cochrane et al., 2008]. With the reticular formation broken, HMP retains in its structure H^{+} reducing the acid diffusion into the enamel decreasing enamel demineralization.

The results of this study cannot be considered definitive because although the chemical model simulates the development of caries under controlled conditions, it still has limitations. All *in vitro* protocols are not able to reproduce the complex intraoral conditions or to mimic solid surface area/solution ratios; the substrate utilized is bovine enamel; the time periods of de- and remineralization are much faster; they are not able to adequately simulate clearance of products and the saliva/plaque fluid composition [Buzalaf et al., 2010]. But, the *in vitro* studies can estimate the role of new anticaries compounds and to screening tests.

It is possible to inhibit enamel demineralization with low-F gels supplementing these gels with 9% HMP, *in vitro*.

5.0 Acknowledgments

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TABLES LEGEND

Table 1: Mean (SD) values of hardness (n =10) analysis according to the groups

Table 2: Concentrations of calcium fluoride (CaF_2-like) and fluoride (FA-Like) present in enamel (formed and retained) after *in vitro* experiment according with the groups

Table 1: Mean (SD) values of hardness (n = 10) analysis according to the groups

Groups	%SH	ΔKHN	ΔIH	
			zone A (5-30 μm)	zone B (40-130 μm)
Placebo	-87.3^{a} (2.3)	10,924.7^{a} (1,559.8)	-	-
9% HMP	-81.3^{d} (5.2)	9,429.3^{d} (430.9)	^{A}426.8^{c} (142.8)	^{B}685.0^{a} (149.5)
4500	-47.2^{b} (2.3)	7,647.6^{b} (674.2)	^{A}1,765.1^{a} (395.5)	^{B}685.1^{a} (152.1)
4500 9% HMP	-33.4^{c} (3.9)	4,637.3^{e} (736.4)	^{A}3,620.5^{d} (596.0)	^{B}1,570.1^{b} (241.9)
9000	-31.8^{c} (4.5)	6,523.1^{c} (538.3)	^{A}2,778.1^{b} (371.5)	^{B}698.1^{a} (147.0)
Acid gel	-33.0^{c} (2.9)	5,348.7^{e} (762.3)	^{A}3,633.8^{d} (498.3)	^{B}979.0^{c} (273.6)

Distinct superscript lowercase letters indicate statistical significance in each columns (%SH, ΔKHN and ΔIH; Student-Newman-Keuls test, $p < 0.05$). Distinct superscript capital letters indicate the differences between zones A and B in each line (Student-Newman-Keuls test, $p < 0.05$).

a: %SH: percentage surface hardness after pH cycling - KHN

b: ΔKHN: integrated loss of subsurface hardness- KHN x μm

c: ΔIH: positive values denote higher integrated hardness and vice versa Depth- KHN x μm

Table 2: Concentrations of calcium fluoride (CaF_2-like) and fluoride (FA-Like) present in enamel (formed and retained) after *in vitro* experiment according with the groups

Groups	CaF_2-like (µg F/cm^2)		FA-like (µg F/mm^3)	
	Formed	Retained	Formed	Retained
Placebo	$^{A}0.11^{a}$ (0.05)	$^{B}0.16^{a}$ (0.05)	$^{A}0.07^{a}$ (0.01)	$^{B}0.56^{a}$ (0.07)
9%HMP	$^{A}0.11^{a}$ (0.12)	$^{B}0.19^{a}$ (0.05)	$^{A}0.06^{a}$ (0.01)	$^{B}0.56^{a}$ (0.10)
4500	$^{A}0.63^{b}$ (0.20)	$^{B}0.21^{a,c}$ (0.05)	$^{A}0.09^{b}$ (0.02)	$^{B}0.61^{a}$ (0.11)
4500 9%HMP	$^{A}1.10^{d}$ (0.51)	$^{B}0.34^{b}$ (0.11)	$^{A}0.09^{b}$ (0.05)	$^{B}0.63^{a}$ (0.10)
9000	$^{A}2.33^{c}$ (1.02)	$^{B}0.22^{a,c}$ (0.05)	$^{A}0.10^{b}$ (0.03)	$^{B}0.60^{a}$ (0.09)
Acid gel	$^{A}4.01^{e}$ (2.00)	$^{B}0.31^{c,b}$ (0.07)	$^{A}0.10^{b}$ (0.03)	$^{B}0.59^{a}$ (0.15)

Lowercase letters indicate differences between groups in each analysis (CaF_2-like and FA-like) and capital letters indicate the differences between CaF_2-like formed and retained and FA-like formed and retained (Student-Newman-Keuls test, $p < 0.05$).

FIGURE LEGEND

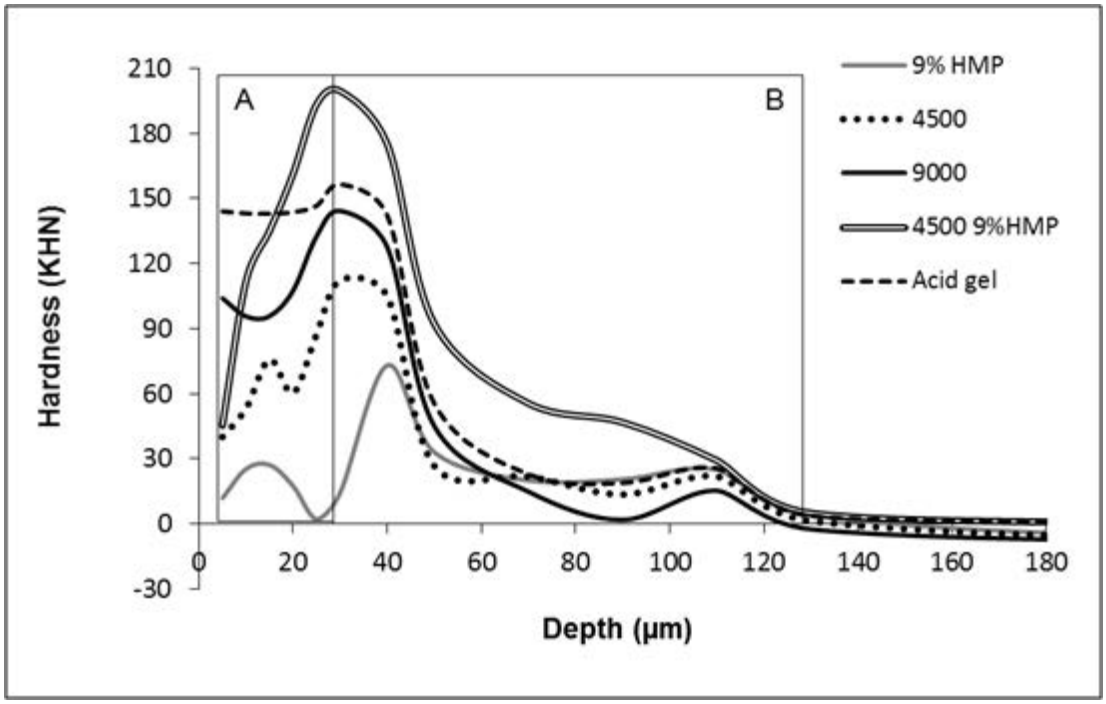

Figure 1: A - Differential hardness profiles (hardness vs depth) calculated by subtracting no fluoride gels profiles from placebo. B - Differential hardness profiles calculated by subtracting fluoride gels profiles from 4500 group (Table 1) (Anova test, $p > 0.05$).

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Anexos

ANEXO A

Instructions for Authors

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Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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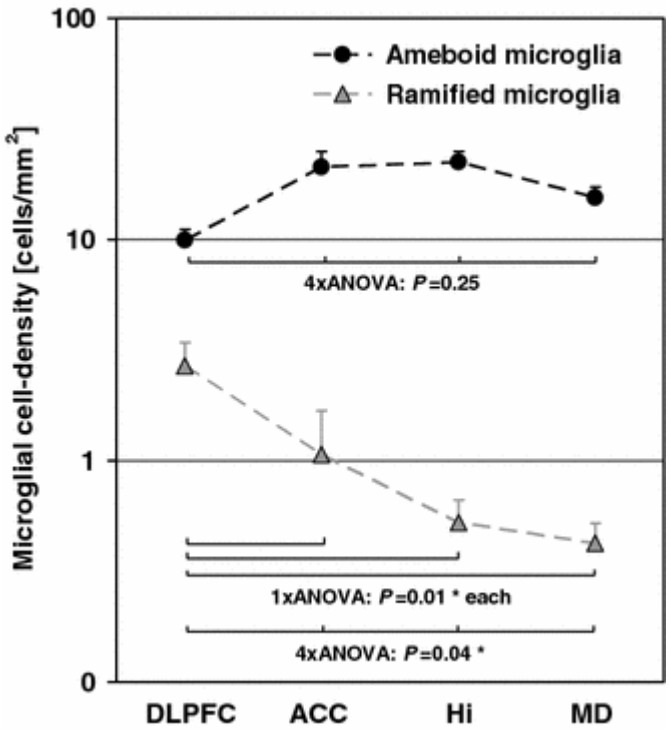

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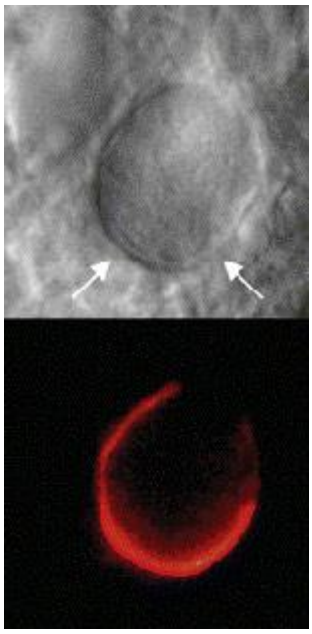

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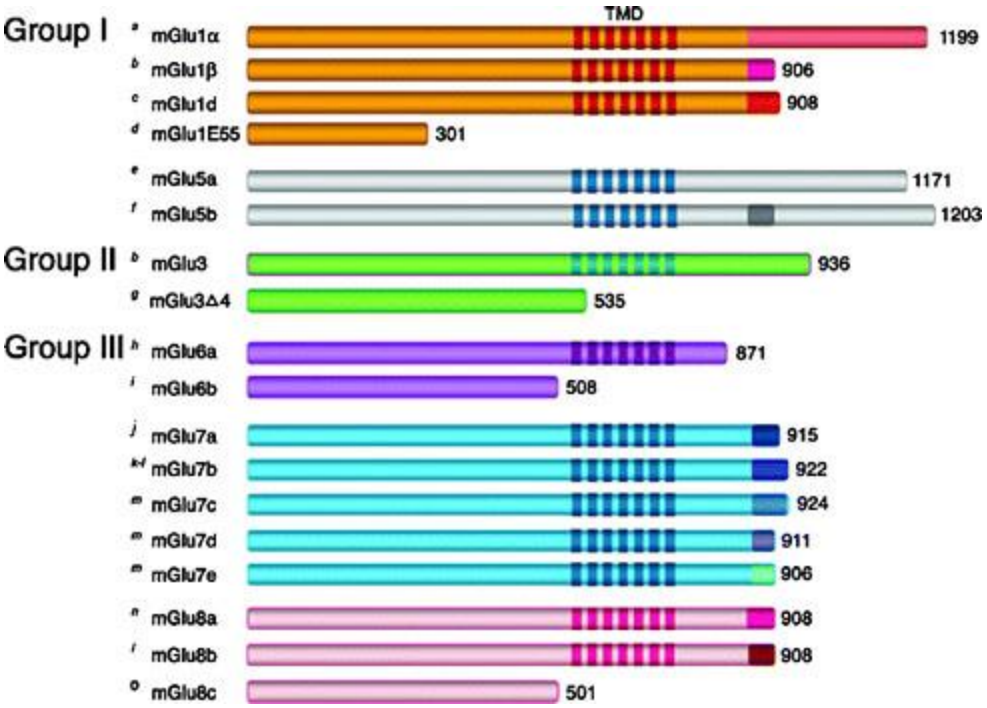

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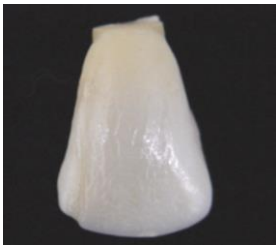
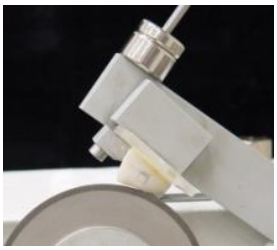
ANEXO B

CONFECÇÃO DOS BLOCOS DE ESMALTE BOVINO (4X4mm)

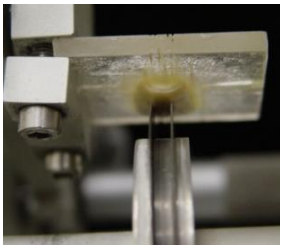


1. Coroa do dente bovino incisivo central inferior, separada da raiz por meio de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob refrigeração (água destilada/deionizada).

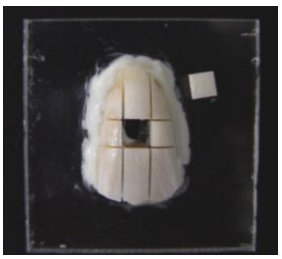


2. Secção da coroa utilizando disco diamantado (série 15 HC Diamond - n. 11-4244 Buehler) separando a superfície vestibular da lingual.

3. Face vestibular fixada na placa de acrílico.



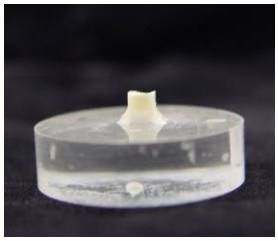
4. Secção da face vestibular no sentido longitudinal, na porção mais plana, utilizando-se 2 discos diamantados (série 15 HC Diamond –n. 11-4243 Buehler), montados em cortadeira sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 4 mm de espessura. Em seguida, foi realizado o corte no sentido transversal.



5. Fragmento vestibular do dente bovino, fixado sobre placa de resina. Ao lado, bloco de esmalte dentário.

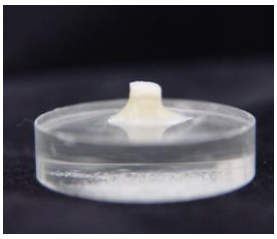
ANEXO C

LANIFICAÇÃO DA DENTINA E POLIMENTO DO ESMALTE



1. Bloco de esmalte fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 11 mm de espessura), com auxílio de cera pegajosa (Kota Ind. e Com. LTDA), com a superfície dentinária voltada para cima.

2. Ajuste da dentina para obtenção de superfícies paralelas entre esmalte e dentina, utilizando Politriz Beta – Grinder – Polisher e Vector Power Head (Buehler, Lake Bluff, IL, EUA) e lixas de granulação 320 (Carbimet Paper Discs, 30-5108-320, Buehler), durante 30 s sob baixa rotação e refrigeração.



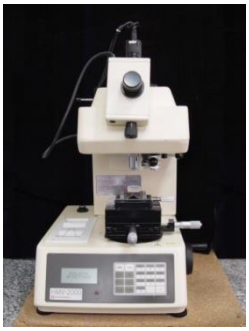
3. Blocos fixados com a superfície do esmalte voltada para cima para serem polidos.

Sequência do polimento de esmalte:

- ✓ Pedra-pomes, água deionizada e taça de borracha montada em contra-ângulo em baixa-rotação.
- ✓ Lixas de granulação 600 (20 s) e 1200 (30 s) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/ deionizada por 2 min, entre cada lixa;
- ✓ Acabamento final com disco de papel feltro TEXMET 1000 (Buehler Polishing Cloth) durante 1 min com suspensão de diamante 1 micron base-água (Buehler);
- ✓ Lavagem durante 30 s com jato de água deionizada;
- ✓ Limpeza em lavadora ultrassônica Modelo 2110 (Branson, Danbury CT, EUA) com água destilada/ deionizada (2min).

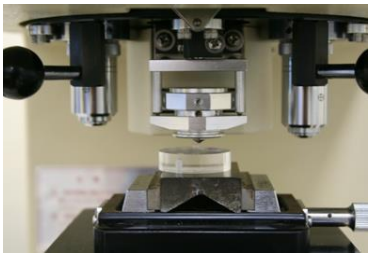
ANEXO D

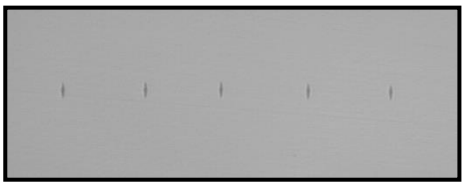
ANÁLISE DA DUREZA DE SUPERFÍCIE DO ESMALTE



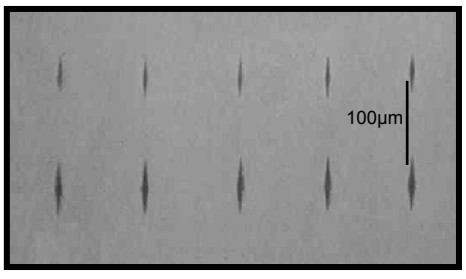
1. Microdurômetro Shimadzu Micro Hardness Tester HMV-2000 (Shimadzu Corporation - Kyoto-Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, EUA).

2. Bloco de esmalte sendo submetido à leitura no microdurômetro, carga estática de 25 g e tempo de 10 s.





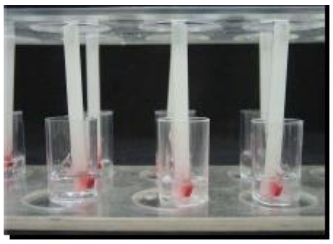
3. Fotomicrografia das impressões (SH*i*). (Aumento: 100x)



4. Fotomicrografia das impressões (SH*i,* SH*f*). (Aumento: 100x)

ANEXO E

CICLAGEM DE pH (Des>Re)



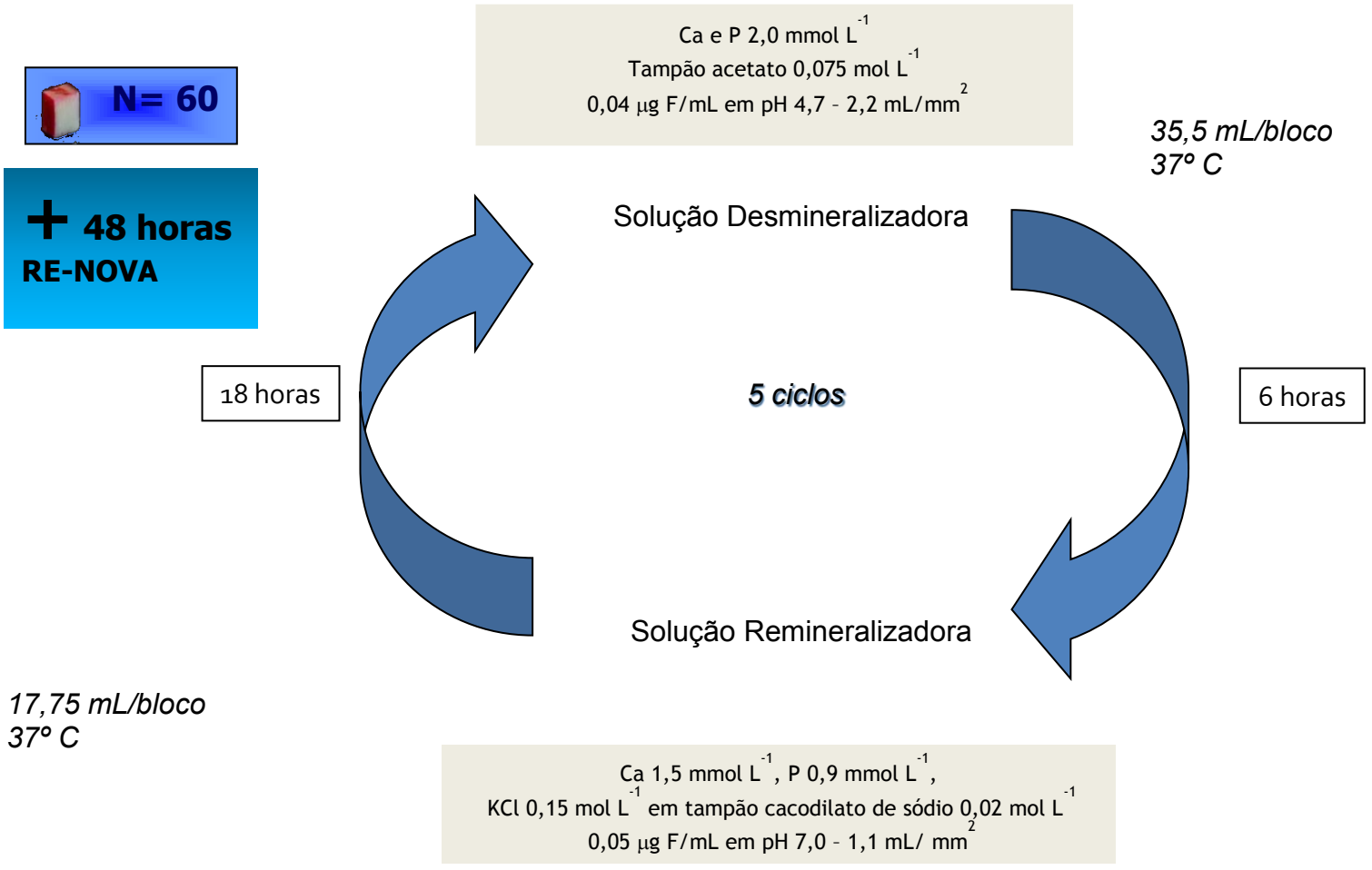
1. Tratamento dos blocos com 3g/gel, por 1 minuto, antes da ciclagem de pH.

2. Lavagem dos blocos de esmalte antes e após o tratamento, durante 30 segundos com água deionizada.

3. Estufa para cultura bacteriológica (Olidef cz Ribeirão Preto – SP, Brasil) utilizada para manter os blocos de esmalte nas soluções de Des e Re em temperatura 37℃, durante o período da ciclagem.

ANEXO F

ESQUEMA REPRESENTATIVO DA CICLAGEM DE pH (DES>RE)~



Vieira et al., 2005.

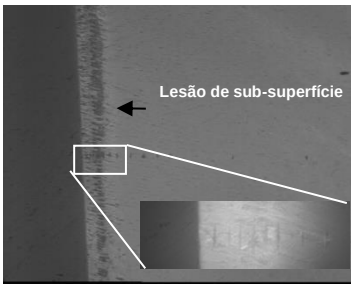
ANEXO G

ANÁLISE DA DUREZA EM SECÇÃO LONGITUDINAL

1. Embutidora metalográfica (AROTEC PRE 30S) – utilizada para inclusão dos blocos de esmalte em 5 g de resina acrílica (Buehler Transoptic Powder, Lake Bluff, Illinois, EUA), pressão de 150 Kgf/cm^2, tempo de aquecimento de 7 min e mais 7 min de resfriamento. Os blocos foram fixados em posição com cola adesiva (Super Bonder – Loctite).

2. Corpo de prova – plano longitudinal voltado para a superfície da resina acrílica.

3. Microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, EUA e Mitutoyo Corporation, Kanagawa, Japão), com penetrador tipo Knoop, acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff,EUA).



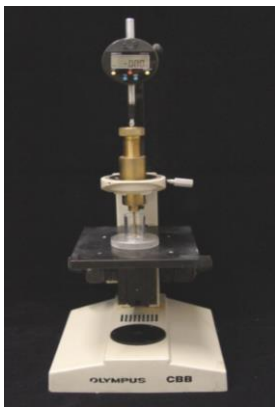
4. Fotomicrografia das impressões. (Aumento: 1000x)

Seqüência do polimento de esmalte:

- ✓ Lixas de granulação 320 (1 min), 600, 800 e 1200 (2min) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/ deionizada por 2 min, entre cada lixa;
- ✓ Acabamento final com disco de papel feltro Microcloth Supreme PSA (Buehler) durante 2 min com suspensão de diamante 1/4 micron base-água (Buehler);
- ✓ Lavagem durante 30 s com jato de água deionizada;
- ✓ Limpeza em lavadora ultrassônica utilizando água destilada/deionizada (2 min).

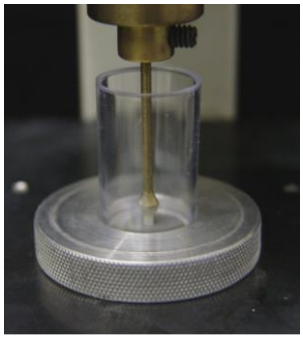
ANEXO H

ANÁLISE DO CONTEÚDO DE F NO ESMALTE



1. Bloco fixado em mandril para peça reta e montado em um microscópio modificado com um micrometro (Pantec, São Paulo, Brasil).

2. Bloco de esmalte adaptado ao mandril, sendo submetido à microabrasão, com desgaste de 100 µm, para análise do F.



3. Após desgaste, pó de esmalte presente na lixa adaptada em frascos de poliestireno cristal (J - 10, Injeplast, Brasil).

4. Para análise do conteúdo de F no esmalte utilizou-se:

A- Eletrodo específico Orion 9409-BN (Orion Research, Inc., Beverly, MA, EUA).

B- Microeletrodo de referência (Analyser Comércio e Indústria LTDA, São Paulo, SP).

C- Analisador de íons Orion 720A (Orion Research, Inc.).

ANEXO I

DOSAGEM DE FLUOR NOS GEIS

Valores de fluoreto (FI) iônico nos géis experimentais e comercial.

(Média (dp), n=3)

Gel	FI (ppm F)
Placebo	67,7 (12,4)
9% HMP	88,8 (8,6)
4500	4681,1 (8,6)
9000	9067,7 (98,4)
4500 9%HMP	4726,4 (145,4)
Gel ácido	10900,2 (219,6)

ANEXO J

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